Fosmid (40 kb) Library Creation Protocol

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Summary

To construct a randomly sheared, non-biased fosmid library containing 40 kb inserts.

Materials & Reagents

Materials/Reagents/Equipment	Vendor	Catalog Number
Disposables		
Phase Lock Gel	Eppendorf	0032 005.101
Clear Round Bottom 2 ml tubes	Eppendorf	22 36 335-2
Falcon 14mL Polypropylene tube	Becton Dickinson	352059
LB chlorenphenicol 12.5 (no X-Gal) plates	Teknova	L4013
Reagents		
Copy Control Fosmid Kit	Epicentre	CCFOS110
MaxPlax Lambda Packaging Extract	Epicentre	MP5120
SYBR Gold gel stain	Molecular Probes	S-11494
1M $MgCl_2$	Ambion	9530G
1M $MgSO_4$	Sigma	M-3409
Chloroform	Sigma	C-2432
Nuclease-free Water	Ambion	9930
0.5M EDTA	Ambion	9262
1M Tris-HCl	Sigma	T-3038
Low Melting Point Agarose	Invitrogen	15517-022
AgarACE	Promega	M1743
Phenol	Sigma	P4557
5M NaCl	Ambion	9759
Pellet Paint	Novagen	69049-3
100% EtOH		
SOC Medium	Teknova	0166-10
LB Broth	Teknova	L8000
Equipment		
Hamilton Syringe		
Hydroshear Wash Kit	Gene Machines	HSH-KT1
-80C freezer		
-20C freezer		
Juan vacuum centrifuge		
Microcentrifuge (13,500 rpm)		

Equipment	
Chef-DR III System	BioRad
500ml Flasks	
Dark Reader Transilluminator	
DNA Speed Vac	Savant

Procedure

Syringe Shearing:

1. Transfer to **well-labeled** Eppendorf tube ~ 20 µg of non degraded DNA; Final volume 60 µl in TE.
   To concentrate, Dry DNA down to 60 µl *if needed.*

2. Shear by hand with Hamilton Syringe.
   a. Wash syringe before and after shearing each sample with 3 X's 0.2M HCl, 2 X's 0.2M NaOH, and 5 X's TE.
   b. Volume = 60 µl
   c. # of passes thru syringe = 4 cycles
   d. Speed = medium

3. Collect sample and **place on ice** immediately.

Blunt End Repair:

1. To 52 µl of sheared DNA, add (Reagents supplied in both the Epicentre fosmid cloning kit and Epicentre End-it kit):

	1 Rxn
Buffer ----------	8 µl
dNTPs ----------	8 µl
ATP ----------	8 µl
**Vortex	
ER enzyme mix ----------	4 µl
Total volume	**80** µl

2. Cap **well-labeled** tube, Vortex, and Spin Down.

3. Incubate:
   a. Room Temp. for 45 minutes
   b. While waiting pour 1% LMP gel using 1x TBE (no EtBr) at 4°C.
   c. After 45 min incubation of sample, add 8 µl loading dye and 3 µl 80% glycerol, then heat at 70°C for 10 minutes.
   d. Keep on ICE until ready to load on gel (gel needs about 50 min to solidify).

Size Fractionation/Gel Separation:

Pulse Field Conditions:

a. % Agarose---- 1.0% Low Melting Point Agarose (no EtBr)
b. Buffer---------- 1x TBE
c. Temperature-- 14^0C
d. Voltage--------- 4.5 V/cm
e. Pulse---------- initial 1.0 – final 7.0 sec
f. Run Time----- 13 hrs
g. Angle---------- 120^0

1. Load sample onto 1% LMP agarose gel (1x TBE).
2. Run gel overnight with above parameters (run with size "Marker 2" and with "T7").
3. Remove gel from pulse field platform.

Image Gel and Cut out DNA

1. Prepare SYBR gold staining solution. (20 µl of SYBR gold into 200 mL of 1X TBE).
2. Stain gel for 15 min on shaker.
3. Use Dark Transilluminator to view bands when cutting.
4. Cut out 40 kb band (the band above the 23 kb marker II band) and band at bottom of well. Place in **well-labeled** 2 ml round bottom Eppendorf tube.
5. Image gel after bands have been cut out. Save file.

Gel Digestion:

1. Place tubes at 70°C to melt gel **completely** (approximately 5 to 20 minutes).
2. Place at 42°C to equilibrate for 3 to 5 minutes.
3. Divide melted gel slice into tubes with no more than 500 µl in each.
4. Add 6-8 µl AgarACE to each tube.
5. Mix well and Spin down.
6. Incubate at 42°C for 20 minutes.

Phenol Extraction:

1. Prepare phase lock tubes, spin at 10,000 RPM for 2 min.
2. Measure sample volume.
3. Add an equal amount of phenol.
4. Vortex well for 15-30 sec.
5. Add to **well-labeled** phase lock tubes.
6. Spin tubes for 5 minutes at 10,000 RPM.
7. Pull off (top) aqueous layer into new **well-labeled** 2 ml round bottom tube.

EtOH ppt:

1. Measure sample volume.
2. Add 1/10 volume of 1M NaCl, 1.5 µl pellet paint, and 2.5 volumes of 96% EtOH.
3. Vortex well and place tubes at –80°C for at least 30 minutes.
4. **Pre-chill microcentrifuge to 4ºC,** this takes at least 15 min.
5. Spin at 13,500 rpm for 20 minutes at 4°C.
6. Discard supernatant, keep an eye on the pink pellet.

7. Wash pellet with 200 µl 96% EtOH.
8. Pull off supernatant being careful of "the wiley pellet" (combine pellets of same sample here).
9. Dry pellet in vacuum for 5 min. at medium heat.
10. Resuspend pellet in 55 µl T0.1E; vortex and spin down sample.
11. Place at 50°C for 5 minutes to fully resuspend pellet.
12. QC 2 µl of sample on 1% agarose gel for size and concentration.
13. Store at –20°C if needed.

Ligation Reaction:

1. Combine the following in a **well-labeled** tube (*reagents provided in fosmid kit*):

1x	
2.0 µl	10x Ligation Buffer
2.0 µl	ATP 10mM
2.0 µl	pCC1FOS vector
12.0 µl	insert DNA (40 kb)
2.0 µl	DNA Ligase
20 µl	total volume

2. Incubate at room temp for 2 hours.
3. Heat kill 10 min at 70 degrees.
4. Chill on ice for 10 min.
5. Store at –20°C until needed.

Day 1 Culture (*day before plating*):

1. In a flask add 100 mL of LB Broth and 1 mL of 1M $MgSO_4$
2. Add 5 µl of *E. coli* cells (EPI300 stored in –80 C).
3. Place culture in shaker at 37°C overnight (14-18 hrs) @ 200 rpm.

Day 2 Culture (*day of plating*):

1. In a flask add 100 mL of LB Broth and 1 mL of 1M $MgSO_4$
2. Add 5 mL of Day 1 Culture from previous day (make sure Day 1 is viable).
3. Place culture in shaker at 37°C for 4-6 hours @ 200 rpm.

Packaging:

1. When thawed (thaw on Ice), add 25 µl of packaging extract to 10 µl of ligation in a **well-labeled** tube.
2. Mix gently with tip and Incubate at 30°C for 90min.
3. Add 25 µl more of packaging extract (thaw on Ice).
4. Mix gently with tip and Incubate at 30°C for 90min.
5. Add Phage Dilution Buffer to get 1000 µl final volume in each tube.
6. Add 25 µl of chloroform to each tube.
7. Mix and store at 4°C until ready to plate (can store for ~ 30 days).

Plating:

1. Before the plating, prepare one **well-labeled** LB - chloramphenicol (@12.5 µg/ml no X-gal needed) agar plate per library by letting it warm to 37°C in incubator to dry.

2. Add 25 µl of packaging/phage dilution buffer solution to 250 µl of Day 2 Culture.
3. Incubate at 37°C for 20min.
4. Plate all (275 µl) onto the chloramphenicol plate using 250 µl of SOC.
5. Incubate the plates in 37°C incubator for 16-18 hrs.
6. Count colonies and determine the complexity of ligation reaction (total # of colonies in ligation).

Reagent/Stock Preparation

T0.1E

10 µl 0.5M EDTA
500 µl 1M Tris-HCl
49.49 mL Nuclease-free Water
pH 8.0

1M NaCl

10 mL 5M NaCl
40 mL Nuclease-free Water

96% EtOH

20 mL distilled water
480 mL EtOH (200 proof)

Phage Dilution Buffer

1 mL 1M Tris-HCl (pH 8.3)
2 mL 5M NaCl
1 mL 1M $MgCl_2$
96 mL distilled water